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# Identification of Proteins by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry Using Peptide and Fragment Ion Masses

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## 1. Introduction

Methods for the identification of proteins have advanced dramatically this decade through the introduction of mass spectrometric techniques and instrumentation sensitive enough to be applicable to biological systems *(1)*. The two mass spectrometric techniques that have provided these advantages are electrospray-ionization mass spectrometry (ESI-MS) and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) (*see* Chapters 52, 54, and 55).

This chapter will deal with the identification of gel-separated proteins, whether or not they have been electroblotted to a PVDF-based membrane. Therefore, we will detail methods used to generate peptide fragments from in-gel digests or on-membrane digests from Immobilon-P or Immobilon-CD, using essentially the methods of Moritz et al. *(2)*, Pappin et al. *(3,4)*, and Patterson *(5)* (as modified from Zhang et al. *[6]*), respectively (*see* Fig. 1). The microcolumn sample clean-up approach is a more detailed description of that which we have published previously *(7)*. We will not reiterate the electrophoretic methods required to separate and electroblot the proteins to a membrane or visualize the proteins (*see* Chapters 35–37). MALDI-MS methods for accurate mass determination of the released peptide fragments together with approaches that utilize this peptide-mass data for identification will then be covered.

The principle behind identification of proteins via accurate mass measurements of enzymatically derived peptides relies on the frequency of specific cleavage sites within a protein yielding a set of potential peptide masses that are unique to that sequence entry when compared with all of the others in the

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database. Studies have shown that only four to six peptide masses are required to identify proteins in searches of a database of more than 100,000 entries *(8)*. Masses sufficiently accurate for this purpose are easily obtained using MALDI-MS. The approach requires that the protein to be identified (or a very close sequence homolog) exists in the database. The caveats to this approach and considerations for interpretation of the results will be described.

Finally, we will cover generation and analysis of sequence-specific fragment ions using postsource decay MALDI-MS. Either partially interpreted or uninterpreted fragment ion data can be used in search programs to identify proteins with high accuracy in sequence databases, and the use of this approach will also be described.

## 2. Materials

### 2.1. Equipment

1. Water bath (able to be set at 25°C or 37°C).
2. Sonication bath.
3. Vacuum concentrator (e.g., Speed-Vac, Savant, Farmingdale, NY).
4. Polypropylene microcentrifuge tubes (500 µL).
5. MALDI-MS instrument (linear and/or reflector capability).
6. MALDI-MS instrument fitted with a reflector capable of variable voltage or a curved-field reflector (the example will employ the Kompact MALDI IV—a curved-field reflector MALDI-MS [Kratos Analytical, Ramsey, NJ]).

### 2.2. General Reagents

1. 50% v/v Methanol (MeOH) containing 0.1% v/v aqueous trifluoroacetic acid (TFA).
2. Deionized water ($dH_2O$).

### 2.3. Enzymatic Digestion In-Gel

1. 50 m*M* citric acid.
2. 20 m*M* $NH_4HCO_3$ containing 50% v/v acetonitrile (MeCN).
3. Stock enzyme: Endoproteinase LysC (5-µg vial, sequencing-grade) (Boehringer Mannheim, Indianapolis, IN) (LysC).
4. 20 m*M* $NH_4HCO_3$, 1.0 m*M* $CaCl_2$ (digest buffer).
5. Laboratory rotating mixer.
6. 1% TFA.
7. 60% v/v MeCN containing 0.1% v/v aqueous TFA.

### 2.4. Enzymatic Digestion on Immobilon-CD

1. 20 m*M* Tris-HCl (pH 9.0) containing 50% v/v MeOH.
2. 25 m*M* Tris-HCl, 1 m*M* EDTA (pH 8.0).
3. Stock enzyme (LysC): as for **Subheading 2.3., item 3**.
4. 30% v/v MeCN containing 2.5% v/v aqueous TFA.
5. 60% v/v MeCN containing 2.5% v/v aqueous TFA.

### 2.5. Enzymatic Digestion on Immobilon-P

1. 70% v/v Aqueous MeCN.
2. 25 m*M* $NH_4CO_3$, 1% octyl-β-glucoside, 10% v/v MeOH (digest buffer).
3. Stock enzyme (LysC): as for **Subheading 2.3., item 3**.
4. 50% v/v Ethanol/50% v/v formic acid (HCOOH) (98%) (prepared immediately prior to use).
5. 10% v/v Aqueous MeCN.

### 2.6. Microcolumn Chromatography

1. μ-Guard™ column, 300 μm id × 1 mm, C8 packing, 300-Å wide-pore (LC packings, San Francisco, CA) (*see* **Note 1**).
2. Lite-Touch® ferrules for 1/16-in. od tubing.
3. Inlet tubing, 2 cm, 300 μm id, 1/16-in. od Teflon® tubing (PE-Applied Biosystems Division, Foster City, CA) or similar.
4. Outlet tubing, 2 cm, 0.005-in. id, 1/16-in. od PEEK (Red) (Upchurch Scientific, Oak Harbor, WA).
5. Hamilton syringe, 10 μL with a fixed or removeable beveled needle and Chaney adapter (Baxter Healthcare, Irvine, CA).
6. 1% v/v Aqueous HCOOH (98%) (make fresh weekly).
7. 10% v/v Increments, or as desired, of MeCN to 90% in 1% v/v aqueous HCOOH (98%) (make fresh weekly).
8. MeOH.
9. Parafilm™.

### 2.7. MALDI-MS Reagents

1. α-Cyano-4-hydroxycinnamic acid (4HCCA, 97%, Aldrich, St. Louis, MO) 10 g/L (50 mg/5 mL) in 70% v/v MeCN containing 30% 0.1% v/v aqueous TFA.
2. 33 m*M* α-cyano-4-hydroxycinnamic acid in MeCN:MeOH:$dH_2O$ (5:3:2 v/v) (Hewlett-Packard, Palo Alto, CA).
3. 29 m*M* α-cynao-4-hydroxycinnamic acid in HCOOH:$dH_2O$:2-propanol (1:3:2 v/v) also referred to as formic acid:water:isopropanol, FWI; *see* **item 4**).
4. HCOOH:$dH_2O$:2-propanol (1:3:2 v/v) (FWI).
5. 10 μ*M* bovine insulin β-chain, oxidized (Sigma).
6. Ice-cold 0.1% aqueous TFA.
7. Synthetic peptide $Pro_{14}Arg$.
8. Thionyl chloride (99%+, Aldrich). NB: Only use this reagent in a well-ventilated fume hood, since it reacts violently with water to yield HCl vapor.
9. Methanol, anhydrous (99%+, Aldrich).
10. Heating block capable of maintaining 50°C.

## 3. Methods

### 3.1. Generation of Peptide Fragments

#### 3.1.1. In-Gel Digestion Protocol

This protocol is for proteins visualized in an SDS-PAGE gel using the "reverse-staining" protocol of Ortiz et al. *(9)*. Briefly, this method involves

immersion of the gel in 0.2 *M* Imidazole for at least 15 min after which time the solution is changed to 50 m*M* $ZnCl_2$ until the background becomes opaque. The gel is then rinsed and stored in $dH_2O$ at 4°C. The stain is sensitive to ~100 fmol (loaded on the gel) level, except for heavily glycosylated and sialylated proteins, which are not readily observed *(10)*. The following protocol was originally described for use with Coomassie blue-stained proteins *(2)*. We have not included a reduction and alkylation protocol, but this can be used if required (*see* Chapter 52). The only difference between that method and what is listed below is the use of citric acid for "destaining" or mobilizing the proteins.

1. Clean work area, microfuge tubes, and all utensils that will be used with 50% v/v MeOH/0.1% v/v TFA solution, and let dry (*see* **Note 2**).
2. Identify the band or spot of interest on the gel, and carefully excise (*see* **Note 3**, and **Fig. 1** for schematic). Do not touch the gel except with forceps. Remove the gel piece to a "cleaned" area where the piece (depending on its size) can be chopped into ~1 $mm^2$ or smaller cubes for digestion. Excise a blank region (containing no protein) from the same gel that is equivalent in size to the band or spot of interest to serve as a control (*see* **Note 4**).
3. Place the chopped pieces into a microfuge tube, and wash with a 50-m*M* citric acid solution (usually 200 µL, but depending on band size, more may be used without effect) for 20 min on a rotating mixer. This mobilizes the proteins in the reverse-stained gel. Decant the citric acid solution when completed.
4. Add 500 µL 20 m*M* $NH_4HCO_3$/50% v/v MeCN to the tube containing the gel pieces and again place on the rotating mixer. After 30 min, replace the wash solution with a fresh buffer, and continue to wash for another 30 min. Decant the wash solution when completed.
5. Place the tubes in a vacuum concentrator (e.g., Speed Vac, Savant), and dry the gel pieces completely (minimum of 30 min) (*see* **Note 5**).
6. To the dry pieces in the bottom of the tube, add 1 µL of stock enzyme (LysC at 0.1 µg/µL in $dH_2O$) and 15 µL of 20 m*M* $NH_4HCO_3$, 1.0 m*M* $CaCl_2$. Leave at room temperature for 15 min. Repeat this step until the gel pieces are totally rehydrated. Add 3× more digest buffer than what was used for the rehydration (i.e., if two additions of enzyme and digest buffer were added, in this step, add an additional 90 µL of digestion buffer) (*see* **Note 6**). Gently mix the tube and place in a 37°C water bath for overnight digestion.
7. After brief centrifugation, remove the digest buffer supernatant, add it to a "clean" microfuge tube, and reduce the volume of the supernatant to ~5 µL by vacuum concentration in a Speed Vac. Make every effort not to let the samples dry completely to avoid additional loss of peptides to the tube. Store these tubes for later microcolumn clean-up (*see* **Subheading 3.2.**). To the gel pieces now add 200 µL of 1% v/v TFA and sonicate in a warm (~37°C) sonication bath for 30 min (*see* **Note 7**).
8. Again, after a brief centrifugation, remove the buffer, place it in a "clean" microfuge tube, and reduce the volume to 1–2 µL without drying (if possible).

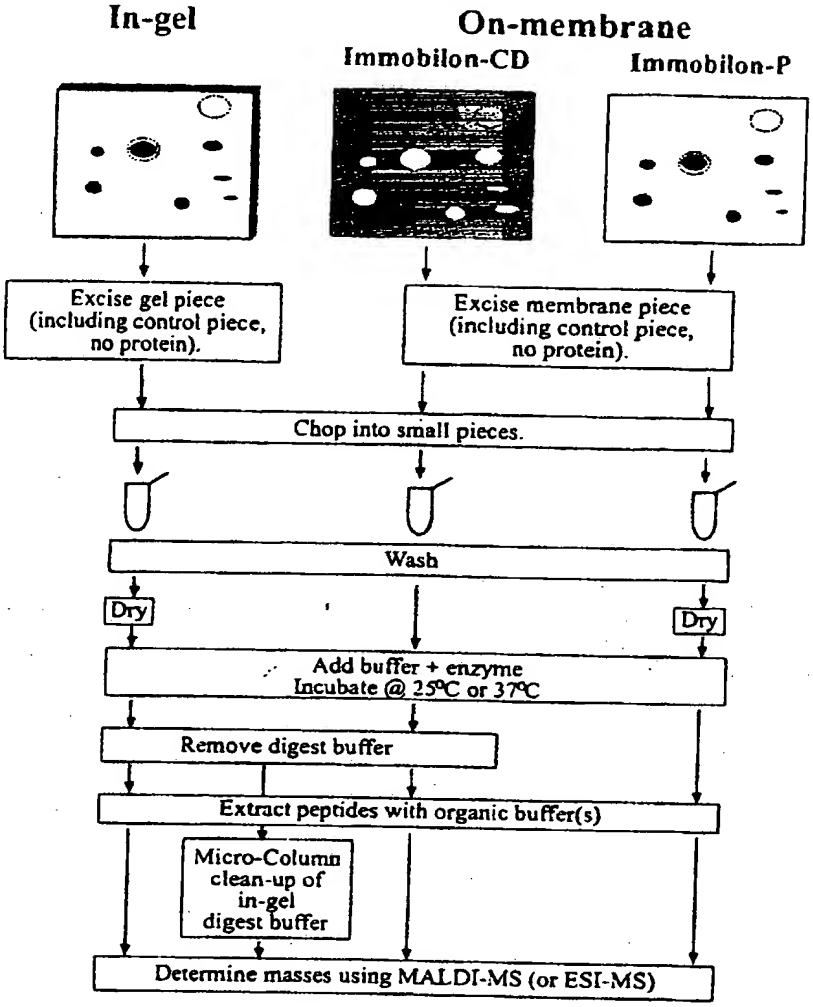


Fig. 1. Flowchart outlining methods for obtaining peptides from gel-separated proteins by either digestion in-gel or on-membrane for subsequent MS analysis. In all cases, the spot (or band) of interest, together with a control piece containing no protein, is excised and subjected to digestion and extraction of the peptides. For the Immobilon-CD protocol, the washing and chopping into small pieces steps can be reversed.

These samples are now ready for MALDI-MS analysis (*see* **Notes 8** and **9**). Next, add 200 µL of 60% v/v MeCN/0.1% v/v TFA to the microfuge tube containing the gel pieces and again place in a warm (~37°C) sonication bath for 30 min (*see* **Note 7**).

9. After a brief centrifugation, remove the buffer, place it in a clean microfuge tube, and reduce the volume to 1–2 µL without drying completely (if possible). These samples are now ready for MALDI-MS analysis (*see* **Note 10**).

### 3.1.2. Immobilon-CD Digestion Protocol

Proteins are generally visualized on Immobilon-CD using a commercial negative stain kit (Immobilon-CD Stain Kit, Millipore, Beverly, MA) that yields a purple background with white areas indicating the presence of protein. The sensitivity of the stain is about 0.5 pmol of protein loaded on the gel *(10)*.

1. Clean work area, microfuge tubes, and all utensils that will be used with a 50% v/v MeOH/ 0.1% v/v TFA solution, and let dry.
2. Identify the band or spot of interest on the wet membrane and carefully excise (*see* **Note 11** and **Fig. 1** for schematic). Do not touch the membrane. Place the membrane pieces into separate microfuge tubes, and wash in 200 µL of 20 m*M* Tris-HCl (pH 9.0), 50% MeOH four times prior to any further manipulation to remove any residual SDS from the membrane.
3. Remove the membrane piece to a cleaned area where the piece (depending on its size) can be cut into ~1 $mm^2$ or smaller squares for digestion. Excise a piece of blank region (containing no protein) from the same membrane of equivalent size as the band or spot of interest to serve as a control (*see* **Note 4**). Keep the membrane pieces wet while cutting them with a drop of $dH_2O$ if necessary.
4. Add the diced membrane pieces to a new 500-µL microfuge tube and add 2 µL (for a membrane piece of ~6 $mm^2$) or 10 µL (for a membrane piece of ~20 $mm^2$) of 25 m*M* Tris-HCl, 1 m*M* EDTA (pH 8.0). Then add either 0.4 or 2 µL of Endo Lys-C (5 µg vial reconstituted in 50 µL deionized water) for the 6- or 20-$mm^2$ membrane pieces, respectively. Incubate at 25°C for at least 20 h (*see* **Note 12**).
5. Remove the digest solution containing any passively eluted peptides and store in a microfuge tube. Extract the membrane pieces with 3 or 10 µL (6- or 20-$mm^2$ membrane pieces, respectively) of 30% MeCN/2.5% TFA. After vortexing for at least 30 s, centrifuge the samples, remove the extract, and store separately in another microfuge tube. Repeat the procedure using the same volume of 60% MeCN/2.5% TFA with sonication for 3 min. The three aliquots are now ready for MALDI-MS analysis, preferably on the same day (*see* **Note 8**).

### 3.1.3. Immobilon-P Digestion Protocol

The proteins can be visualized on Immobilon-P using Coomassie blue or Sulforhodamine B as described *(4)*. The sensitivity of Coomassie is about the same as that for Immobilon-CD, whereas Sulforhodamine B can visualize proteins to the ~100-fmol level.

1. Clean work area, microfuge tubes, and all utensils that will be used with a 50% v/v MeOH/0.1% v/v TFA solution, and let dry.
2. Identify the band or spot of interest on the dry membrane, and carefully excise (*see* **Note 10** and **Fig. 1** for schematic). Do not touch the membrane. Remove the sample to a cleaned area where the piece (depending on its size) can be cut into ~1-$mm^2$ or smaller squares for digestion. Excise a piece of blank membrane (containing no protein) the same size as the band or spot of interest to serve as a

control (*see* Note 4). Rehydration of the membrane pieces while cutting them with a drop of 50% v/v MeOH/$dH_2O$ is sometimes helpful.

3. The Coomassie blue-stained membrane pieces are then put into a microfuge tube and washed/destained using 200 µL of 70% v/v MeCN and vortexing. When the bands are totally destained (~30 s), pipet off the solvent and dry the pieces in a vacuum concentrator for 10 min or until completely dry.
4. To the dry membrane pieces, add 1 µL of your stock enzyme (LysC at 0.1 µg/µL in $dH_2O$) and just enough digest buffer ($NH_4CO_3$/octyl-β-glucoside/MeOH) to cover all of the membrane pieces, usually 3–10 µL, depending on the size of the protein band. It is sometimes helpful at this point to use a clean gel loading pipet tip or needle of a syringe to keep the dry membrane pieces in the digest buffer while they rehydrate (they tend to float on the surface of the liquid in the tube). Incubate the sample at 26–27°C overnight.
5. Remove and store the digest buffer separately from the tube (*see* **Note 13**). If necessary, this can be cleaned up later using the microcolumn (*see* **Subheading 3.2.** and **Note 14**). Add 10 µL of freshly prepared 50% v/v ethanol/50% v/v HCOOH, and place in a sonicator for 30 min. After sonication, remove the extract, and dry completely in a vacuum concentrator. Rehydrate in 1–5 µL of 10% v/v MeCN/1% v/v HCOOH and perform MALDI-MS analysis.

### 3.2. Microcolumn Chromatography for Sample Clean-Up

This technique has been developed as a manual chromatographic clean-up step to remove salts and contaminants from samples (digest supernatants), which would be below the detectable limit for normal microbore chromatography (i.e., a few pmol or less loaded on the gel). This quick, easy clean-up step has not only helped the overall recovery of peptides from techniques of this nature, but has also dramatically lowered the effective limit of in-gel and on-membrane digests to low-picomolar to subpicomolar levels *(7–12)*.

1. Assemble the microcolumn by attaching the outlet and inlet tubing to the µ-Guard column using the Lite-Touch ferrules and tighten by hand. The outlet tubing (Red PEEK) is shaved to yield a conical end allowing 3-µL droplets to form. This will not occur with a blunt end.
2. Equilibrate the microcolumn using 30 µL (3 × 10 µL) MeOH using a 10-µL Hamilton syringe to introduce the solution into the inlet tubing (*see* **Note 15**).
3. Clean work area, microfuge tubes, and all utensils that will be used with a 50% v/v MeOH/ 0.1% v/v TFA solution, and let dry.
4. Prepare the digest supernatant for microcolumn clean-up by reducing its volume to 3–10 µL.
5. Equilibrate the column with 30 µL of 1% v/v HCOOH at a flow rate of approx 1–2 µL/s.
6. Using a 10-µL Hamilton syringe, load your sample into the syringe, and attach to the column inlet. Using the same approximate flow rate as with the equilibration, pass the sample over the column, collecting the flowthrough as the "void/wash"

into a microfuge tube. Reload 10 µL of 1% v/v HCOOH back into the same syringe and pass over column. Continue to collect this wash into the void/wash tube (*see* **Note 16**).

7. Rinse the syringe quickly (2–3×) with 30% v/v MeCN/1% v/v HCOOH (*see* **Note 17**). Load 3 µL of 30% v/v MeCN/1% v/v HCOOH into the syringe, and attach to the column inlet. Again at the same approximate flow rate, pass the solvent over the column, collecting the eluant in a microfuge tube labeled 30% MeCN.
8. Repeat **step 7** using 3 µL of 70% v/v MeCN/1% v/v HCOOH, and elute into an appropriately labeled tube.
9. Repeat **step 7** using 6 µL of 90% v/v MeCN/1% v/v HCOOH, and elute into an appropriately labeled tube. The larger elution volume is used to attempt to ensure complete recovery of peptides from the column.
10. Wash the column with 30 µL of 90% v/v MeCN/1% v/v HCOOH.
11. Repeat the series of steps on the next sample.
12. When all samples are finished, clean the column in MeOH (at least 3 × 10 µL) and wrap the column in Parafilm, being sure the inlet and outlet are sealed to avoid drying out.
13. Proceed with MALDI-MS analysis on all fractions collected (*see* **Note 18**).

## 3.3. MALDI-MS

### 3.3.1. Matrix Preparation and Selection

1. Matrix solutions are prepared in small volumes (5 mL or less) so that they will not be stored for excessive periods of time. Add the appropriate quantity of powdered 4HCCA (listed in **Subheading 2.7.**) to the organic solution, and then add the remaining solution(s). The matrix solution is stored in a container (e.g., glass vial or microfuge tube) protected from the light and may need to be centrifuged briefly to pellet any undissolved chemical prior to use (*see* **Note 19**).
2. The matrix 4HCCA listed in **Subheading 2.7.** can be dissolved in different solvents. In general, we use the commercial matrix preparation, but this is for convenience only and we have not found an appreciable difference in spectra obtained with this and the MeCN/TFA solvent mixture, although one should remember that the commercial matrix preparation also has a limited life. However, the FWI mixture (**Subheading 2.7., item 3**) can dramatically change the peptides observed in complex mixtures *(13)*, and this can be particularly useful when both the "standard" and FWI-based matrices are used in parallel, since more peptides can be observed than with either matrix alone.
3. 4HCCA is the most widely used matrix for peptide mixtures (which this chapter is concentrating on), and can also be employed for proteins up to the size of at least serum albumin. However, sinapinic acid is often used to analyze proteins (*see* **Note 20**).

### 3.3.2. Preparation and Loading of the MALDI-MS Target

1. Rinse the MALDI-MS target with MeOH, and wipe dry with a lint-free tissue, or follow the procedure described by the MALDI-MS manufacturer.

2. The simplest MALDI-MS sample preparation method is to add a small aliquot of the sample ~0.3 μL (in the low to subpmol range) to the target well on the sample slide followed by an equal amount of matrix. This solution is mixed in the pipet tip, and then allowed to dry at room temperature. When small volumes are used, drying only takes a few minutes.
3. Other sample preparation methods include mixing an aliquot of the sample and the matrix in a separate microfuge tube prior to loading the mixture on the target well. The sample/matrix mixture can also be subjected to vacuum to assist in even crystallization.
4. After the sample has dried, and hopefully an even crystalline surface is visible, the sample slide is ready for loading into the MALDI-MS instrument and data analysis.

### 3.3.3. Calibration

It is important to ensure that masses measured with the MALDI-MS are as accurate as possible. This can be achieved through "external" calibration where the calibrant is applied to a target well separate from the sample, or by "internal" calibration where the calibrant is mixed with the sample. In either case, the aim is to use ions of known mass, which bracket the sample to be measured. We recommend incorporating a calibrant with every sample set to provide the opportunity for external calibration with each experiment, or at least to confirm that the instrument calibration is stable.

1. Calibration routines are instrument- (software) dependent, but in their simplest form, they employ a two-point calibration using a matrix ion and a large peptide of known molecular mass.
2. Calibration for peptide mixtures can be performed using 0.3 μL of a 10 μ*M* solution of the oxidized B chain of bovine insulin ($MH^+$ 3496.9) dissolved in $dH_2O$ into the target well, followed by 0.3 μL of matrix. A spectrum averaged from at least 50 shots is obtained at a laser fluence just above threshold to ionize the calibrant, in the same manner that sample data is acquired. The signal from the calibrant (oxidized B-chain of bovine insulin), together with either the dehydrated matrix ion of 4HCCA at $MH^+$ 172.2 or the matrix dimer at $MH^+$ 379.2, allows us to bracket many of the peptides generated in a LysC or tryptic digest.
3. The specific software calibration routine is then followed to fit a straight line to these two ions (matrix and calibrant).
4. Internal calibration can be performed after the sample spectrum has been obtained by redissolving the sample in a small volume (~0.2 μL) of calibrant mixed with matrix (e.g., a ratio of calibrant:matrix of 4:1). The amount of calibrant applied needs to yield an intensity equivalent to that of the sample. This can be judged from the intensity of the calibrant in the external calibrant, although ionization suppression can sometimes occur (*see* **Note 21**).

### .3.4. On Probe Sample Clean-up

Occasionally, despite the procedures outlined above, some samples may still ›t yield signals by MALDI-MS. There are a number of on-probe sample clean-

up procedures, but it is not the aim of this chapter to detail them all. The following procedure first described by Beavis and Chait *(14)* is one of the simplest, and is often very effective. It relies on the difference in solubility between the peptide/protein-matrix crystals, and salts and other low-mol-wt contaminants. The salts are soluble in ice-cold acidic solution, whereas the peptide/protein-matrix crystals are not.

1. Add 2–3 µL of ice-cold 0.1% TFA to the dried sample in the target well for ~5 s, and then remove this with the same pipet (or blowing it off with forced air).
2. This can be repeated at least twice (*see* Note 22).

### 3.4. Peptide-Mass Searches

After the accurate peptide masses are obtained from the protein band or spot, they can be used in search programs to determine whether the protein exists in the current full-length protein sequence databases, or translations of nucleotide sequence databases. There are a number of publicly accessible sites on the internet (World Wide Web), which can be used to search these databases. The following is a list of servers currently available together with their affiliations and URL addresses. All supply full instructions on-line concerning to their use.

1. MassSearch from CBRG, ETHZ, Zurich: http://cbrg.inf.ethz.ch/subsection3_1_3.html.
2. MOWSE search program from SEQNET, Daresbury: http://www.dl.ac.uk/SEQNET/mowse.html.
3. MS-Fit (and MS-Tag) from the University of California, San Francisco: http://rafael.ucsf.edu/MS-Fit.html.
4. Peptide Mass Search from Max Delbruck Center for Molecular Medicine, Berlin: http://www.mdc-berlin.de/~emu/peptide_mass.html.
5. PeptideSearch from EMBL, Heidelberg: http://www.mann.embl-heidelberg.de/Services/PeptideSearch/PeptideSearchIntro.html.
6. ProFound (and PepFrag) search program from Rockefeller University, New York: http://chait-sgi.rockefeller.edu/cgi-bin/prot-id/1/1.

It is essential to obtain the most accurate masses possible. Therefore, we recommend internal calibration for such analyses as described above (i.e., we obtain externally calibrated spectra prior to adding a small amount of calibrant to the sample).

The various peptide–mass search programs each have their own idiosynchrasies, but all require a set of peptide masses (together with a stated tolerance or mass accuracy), the enzyme or chemical reagent used to generate the peptides, whether the cysteine residues have been modified (i.e., carboxylmethylated, and so forth), and whether missed cleavage sites should be considered. Additional input to the program can include modifications to other specified amino acid residues (e.g., methionine sulfoxide), peptide masses

following deuteration (an amino acid composition-dependent mass increase), a selection of which database(s) to search, whether the search should be restricted to a subset of proteins whose intact mass falls within a specified range around the mass of the unknown protein of interest, and the species from which the sample was derived (for reviews, *see* **refs.** ***1***, ***15***, and ***16***). The Daresbury program can use further information on individual peptides, such as partial composition and partial sequence. It should be noted that electrophoresis can induce artifactual modifications, e.g., acrylamide adducted to free cysteines and the *N*-terminus, and methionine oxidation (*see* **Note 23**).

Most programs will yield some result given a set of input peptide masses even if only a few masses are found to match. Rarely do all of the input peptide masses match with the top-ranked candidate. Therefore, it is critical to attempt to determine how these peptide masses arose. The following is a list of possibilities for peptide masses that are not matched with the top-ranked candidate:

1. The correct protein was identified and the nonmatching peptides are owing to either posttranslational modification (including artifactual) or processing (*see* **Note 24**).
2. The correct protein was matched, but some peptides were derived following either unspecific cleavage of the protease or specific cleavage from a contaminating protease (*see* **Note 25**).
3. The correct protein was identified, but was part of a mixture of two or more proteins (*see* **Note 26**).
4. A homolog (or processing/splice variant) from the same or a different species was identified (*see* **Note 27**).
5. The result is a false positive (*see* **Note 28**). The possibility should not be overlooked that the real protein does not exist as an entry in the database being searched and may be truly novel.

When possible, it is advisable to attempt to gain further information on the peptides by either chemical treatments or chemical or mass spectrometric sequencing (*see* **Subheading 3.6.**). Of course this is dependent on the quantity of peptide available for further analysis.

### *3.5. Postsource Decay MALDI-MS*

To improve the confidence in the results of a peptide mass search, one needs to determine additional primary structural information on the peptides observed ***(1)***. One means of achieving this aim is to isolate the peptide in the gas phase of the mass spectrometer and induce fragmentation of the peptide and measure the mass of the fragment (or daughter) ions. There are a number of mass spectrometric instrument designs with various ionization sources that allow gas-phase isolated peptides to be fragmented, e.g., a triple-quadrupole MS, an ion-trap MS, and a time-of-flight (MALDI-MS) when the instrument is fitted with either

a variable voltage reflector or curved-field reflector. We will describe the use of the latter type of MALDI-MS instrument, i.e., a curved-field reflector instrument. However, it should be noted that the same principles apply to the use of a MALDI-MS fitted with a variable voltage reflector. Fragment-ion spectra observed in a MALDI-MS instrument are generated by "postsource decay" or metastable fragmentation of the ions during the ionization process (*see* Note 29). This is distinct from "in-source" or "prompt" fragmentation that occurs very early during the ionization (and therefore acceleration) process, and so the fragment ions are resolved in the linear dimension because of their different velocities (e.g., peptides linked by a single disulfide bond have been observed to undergo prompt fragmentation *[17]*). Fragmentation is sequence-specific, and generates the same ion series observed during other types of gas-phase fragmentation, including both single- and multiple-bond cleavages (*see* Note 30). It should be noted that if the peptide carries a posttranslational modification, the bond linking it to the peptide may be weaker than the peptide bonds themselves, so the fragment ions observed sometimes result from loss of the modifying group and little if no peptide bond fragmentation (e.g., O-linked carbohydrate and, sometimes, phosphate on serine or threonine residues).

### 3.5.1. Matrix Selection, Sample Loading, and Calibration

1. The most commonly used matrix for PSD-MALDI-MS is 4HCCA, since this is considered a relatively "hot" matrix, i.e., there is often considerable metastable fragmentation. The two other most common matrices, sinapinic acid (3,5 dimethoxy cinnamic acid) and gentisic acid (or dihydroxy benzoic acid), yield less metastable decay products. Matrix preparation and sample loading are as described in **Subheadings 3.3.1.** and **3.3.2.**
2. PSD-MALDI mass spectra can only be calibrated externally, since the sample is a single molecular species. The manufacturer will have a specific protocol to follow, but we use the calibrant first described by Cordero et al. *(18)*, which is a synthetic peptide of 14 prolines with an arginine C-terminus ($Pro_{14}Arg$)($M_{ave}H^+$ = 1534.8). However, other peptides, such as Angiotensin II, whose fragment-ion spectrum is well known, can also be employed for fragment-ion calibration. The $Pro_{14}Arg$ peptide readily generates strong *y* series ions with a few *b* series ions as well as the proline immonium ion, thereby providing an excellent range of fragment-ions for calibration (*see* **Fig. 2**).
3. Using the Kompact MALDI IV, the reflector is first calibrated with intact molecular ions in the same manner as in linear mode (*see* **Subheading 3.2., step 3**), and then the calibrant peptide is fragmented, and the masses of the fragment-ions observed without any correction are entered into the calibration program. The expected fragment-ion mass for each selected ion is then entered, and the software uses a curve-fitting program to construct a calibration curve for use with other spectra.
4. Although we routinely check the calibration, in our experience, the calibration is very stable. This is expected, since it is an instrument-dependent parameter. The

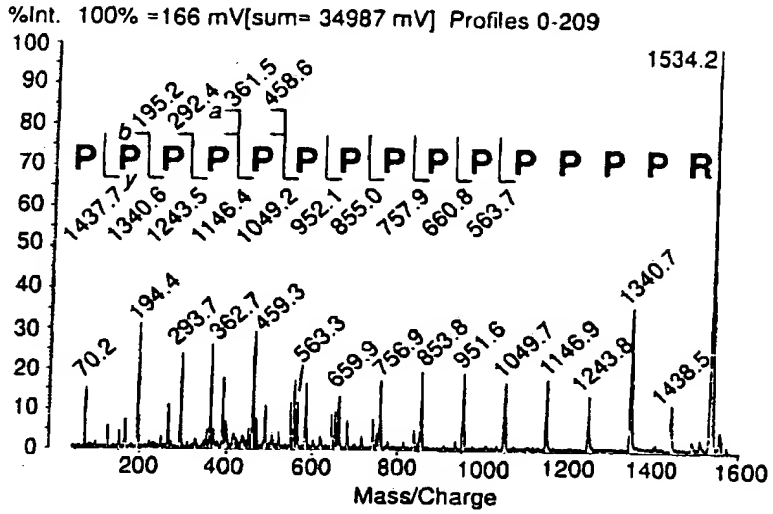


Fig. 2. PSD-MALDI-mass spectrum of $Pro_{14}Arg$ ($M_{ave}H^+$ = 1534.8) using a curved-field reflector MALDI-MS. The observed fragment-ion masses following calibration are listed over the peaks used in the calibration routine. The expected fragment-ion masses for the observed *a*, *b*, and *y* series ions are listed above and below the peptide sequence. The immonium ion of Pro has a mass of 70.1 u. The spectrum was smoothed over four bins, and a baseline subtraction of 40 was used.

same would be expected for MALDI-MS instruments where the reflector is scanned with different set voltages as long as these are stable between analyses.

### 3.5.2. Methyl Esterification as a Means to Assist Fragment-Ion Interpretation

Pappin et al. *(3)* described a simple method for methyl esterification of peptides that can be used both for peptide-mass mapping in linear MALDI-MS and PSD-MALDI-MS (*see* **Note 31**). This method, in most instances, quantitatively esterifies all carboxylic acid groups on peptides to the corresponding methyl esters, thereby increasing the mass by 14 u for each group. A free C-terminus will result in a mass increase of 14 u, and any acidic residues (Asp or Glu) will result in additional 14-u mass increases for the peptide, allowing the number of acidic residues to be calculated. This is of assistance to both peptide-mass and fragment-ion searches as well as for *de novo* interpretation of the fragment-ion spectrum. A simple example using a four-residue peptide of sequence FGSR is shown in **Fig. 3A** and following methyl esterification in **Fig. 3B**.

1. Aliquot a portion, 1–1.5 µL, of the sample (either digest or individual peptide fraction) and dry by vacuum concentration in a 500-µL microfuge tube (e.g., in a SpeedVac) (*see* **Note 32**).

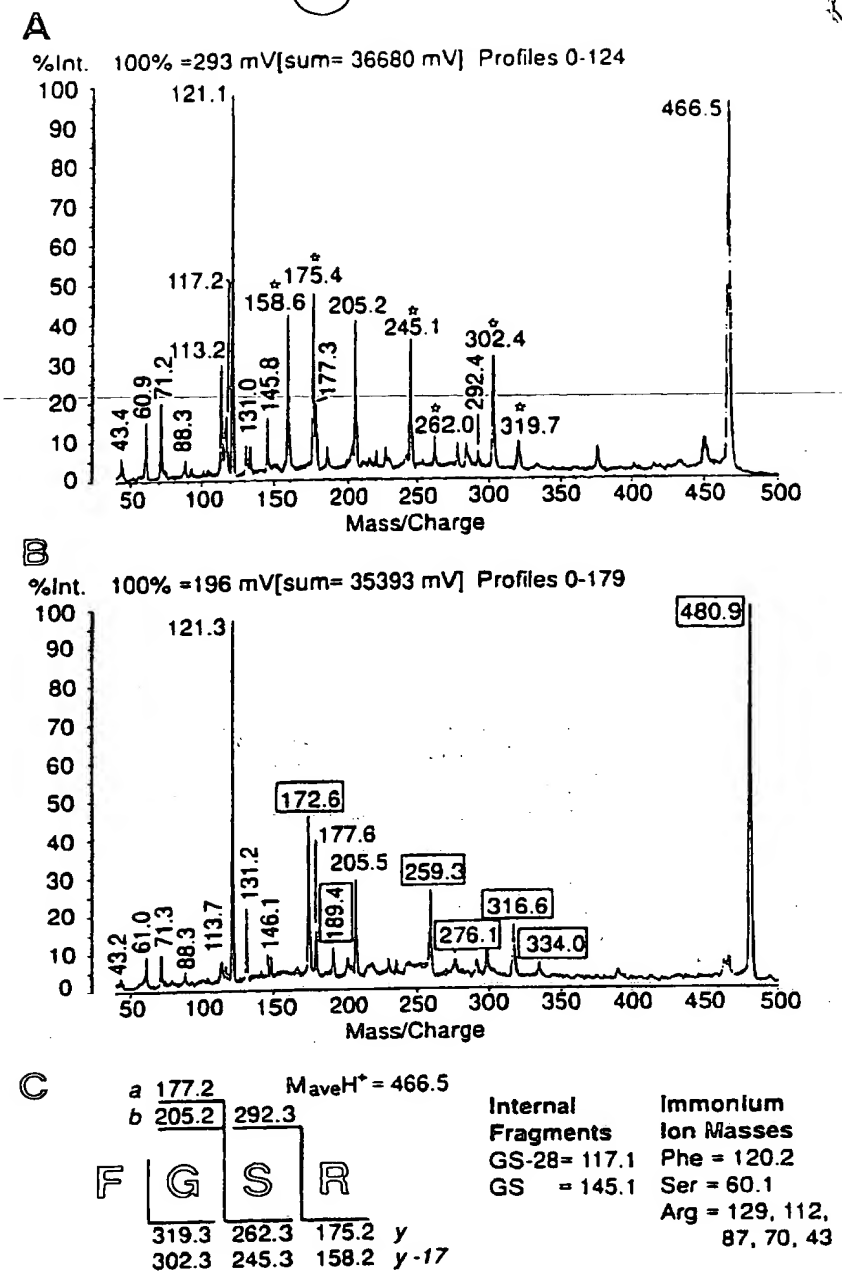


Fig. 3. PSD-MALDI-mass spectrum, using a curved-field reflector MALDI-MS, of a peptide of sequence FGSR with and without methyl esterification. Panel (A) shows the fragment-ion spectrum without methyl esterification, panel (B) with methyl esterification, and panel (C) shows the theoretical fragment-ion masses for the peptide FGSR (corresponding to those labeled in panels A and B). The *y* and *y*-17 (loss of ammonia) series ions are labeled with an * in panel A. All of the ions (*y* and *y*-17 series ions, and the intact molecule) that increase in mass by 14 u following methyl esterification have their observed masses boxed in panel B. Both spectra were smoothed over two bins with no baseline subtraction.

2. Make a 1% v/v thionyl chloride solution in anhydrous methanol (*see* Note 33).
3. Add 3–6 µL of the thionyl chloride solution (3× the volume of the original sample). Cap the tube, and heat at 50°C for 30 min in a heat block (*see* Note 34).
4. Dry the reaction mixture by vacuum concentration, and resuspend with 3 µL of 30% v/v MeCN/1% v/v HCOOH (**Subheading 2.6., item 7**).
5. An aliquot of this solution can be applied to the MALDI sample well, and allowed to dry prior to adding matrix (*see* **Subheading 3.3.2.**).

### 3.5.3. Interpretation of PSD-MALDI-MS Spectra

We do not usually manually interpret PSD-MALDI-MS spectra, instead we use the uninterpreted fragment ions in a computer search (*see* **Subheading 3.6.**). If all of the ions used in the search have not been matched by the program, we then use the matched sequence to see if any unmatched ions can be explained by fragmentations not included in the search program (e.g., some of the search programs do not include internal fragmentations). However, it is certainly possible to interpret manually some PSD-MALDI-MS spectra. The simple spectrum in **Fig. 3** is a good example. The following is a description of how the spectrum could be interpreted, in a manner similar to that described by Kaufmann et al. ***(19)***. The calculated fragment ions for this peptide are shown in **Fig. 3C**. Basically, the strategy is to look for immonium ions and then determining whether an ion series can be identified by mass differences (some of which could correspond to the identified immonium ions). Once a sequence has been formulated, one attempts to correlate all of the observed ions in the spectrum with the sequence.

1. The immonium ion region can often provide information on the amino acid content of the peptide (*see* **Table 1** for immonium ion masses). In **Fig. 3A**, the following ions are observed in this region: 43, 61, 71, 88, 113, 117, 121, 131, 145, and 158. Given the expected mass tolerance (±1 u), these ions could represent immonium ions from Arg (expected 43, 70, 87, 112, and 129), Pro (expected 70), Ser (expected 60), Leu/Ile (expected 86), Phe (expected 120), and Trp (expected 159). However, given the ion intensities—Pro and Leu/Ile too weak, and Trp too strong—these are not likely candidates. The 117 and 145 ions are not matched.
2. The peptide was derived from a tryptic digest, so it would be expected that the C-terminus would be Lys or Arg (although of course there are exceptions, e.g., the C-terminal peptide or a nonspecific cleavage). A strong ion at 175.4 is observed in **Fig. 3A**, which is the expected $y_1$ ion for an Arg C-terminus (expected 175.2). The neutral loss ion at 158.6, which is expected for an Arg, is also present (given the intensity, a more plausible explanation than a Trp immonium ion). If Lys were at the C-terminus, the $y_1$ ion would be 147.
3. We can now look for ions with masses between 232 (175 + 57, Gly) and 361 (175 + 186, Trp), because the next series ion has to have a mass between that of an additional Gly or Trp residue (if no residues are modified). A number of ions are present in this mass range. Since there is an Arg at the C-terminus, the *y*-series

**Table 1**
**Residue Masses of Amino Acids Together with Their Corresponding Immonium Ion Masses[a]**

| Amino acid | Abbreviations—three letter, single letter | Residue mass[b] | Immonium ion mass[b] |
|---|---|---|---|
| Glycine | Gly (G) | 57 | 30 |
| Alanine | Ala (A) | 71 | 44 |
| Serine | Ser (S) | 87 | 60 |
| Proline | Pro (P) | 97 | 70 |
| Valine | Val (V) | 99 | 72 |
| Threonine | Thr (T) | 101 | 74 |
| Cysteine | Cys (C) | 103 | 76 |
| Isoleucine | Ile (I) | 113 | 86 |
| Leucine | Leu (L) | 113 | 86 |
| Asparagine | Asn (N) | 114 | 87 |
| Aspartate | Asp (D) | 115 | 88 |
| Glutamine | Gln (Q) | 128 | 101 |
| Lysine | Lys (K) | 128 | 129, 101, 84[c] |
| Glutamate | Glu (E) | 129 | 102 |
| Methionine | Met (M) | 131 | 104 |
| Histidine | His (H) | 137 | 110 |
| Phenylalanine | Phe (F) | 147 | 120 |
| Arginine | Arg (R) | 156 | 129, 112, 100, 87, 70, 43[c] |
| Tyrosine | Try (Y) | 163 | 136 |
| Tryptophan | Trp (W) | 186 | 159 |

[a]The values were obtained from Jardine *(20)*, and Spengler et al. *(21)*.
[b]All masses are given as average integer values.
[c]Arginine and lysine both exhibit multiple immonium ions, and these are listed (they are not of equal intensity).

ions are expected to exhibit strong neutral losses. The ions at 245.1 and 262.0 could represent a *y* and *y*-17 pair, as could 302.4 and 319.7. In fact, the mass difference between 262.0 and 175.4 is 86.6 (which could correspond to Ser, observed in the immonium ions). The difference between 319.7 and 175.4 (144.3) does not match an amino acid residue. However, the difference between 319.7 and 262.0 of 57.7 could correspond to Gly. This would make the sequence RSG, reading from the C-terminus.

4. The difference in mass from the molecular ion (466.5) and the last assigned *y* series ion (319.7) is 146.8, which matches with Phe (expected mass of 147.2). Thus, the complete *y* series has been observed, and given the immonium ion masses indicated—Arg, Ser, and Phe—the sequence can be assigned with some confidence as FGSR.

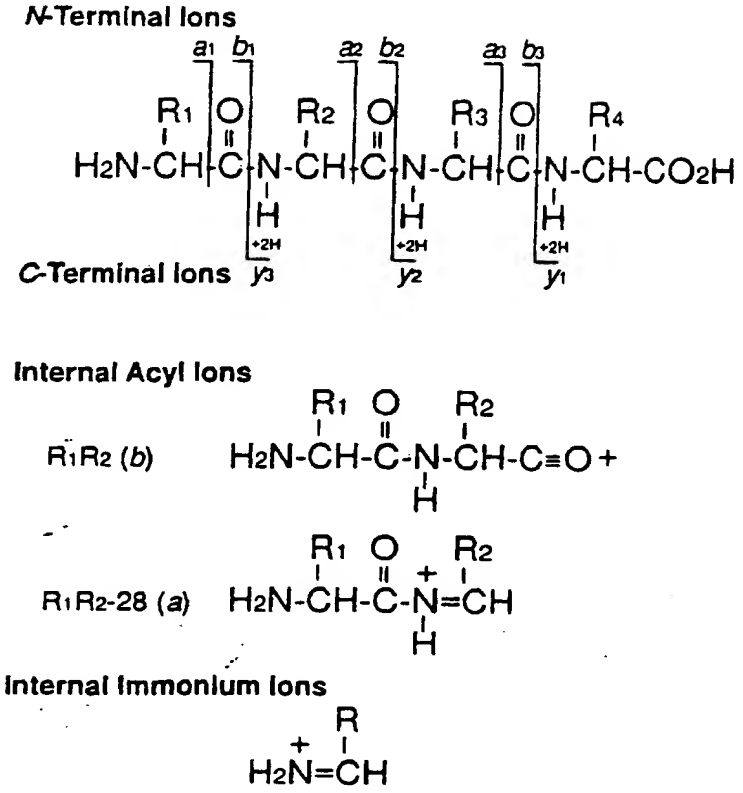


Fig. 4. Fragmentation nomenclature for the most common positive ions observed by PSD-MALDI-MS (after *22,23*). Fragmentation is usually only unimolecular, i.e., only one break occurs in the peptide backbone, and the charge is retained on either the N-terminus (*a* and *b* series ions) or the C-terminus (*y* series ions). However, internal acyl ions and immonium ions can also be formed from multiple fragmentation events. The internal acyl ions are referred to by their amino acid sequence (e.g., $R_1R_2$) (in a form similar to *b* series ions), or their sequence –28 (for those similar to *a* series ions). There are also neutral losses from the internal acyl ions where part of an amino acid side chain is lost (e.g., ammonia [17 u] can be lost from Q, K, and R side chains, and water [18 u] can be lost from S and T side chains). Similar losses can also occur from the molecular ion (a very good list can be found at the MS-Tag WWW site, http://rafael.ucsf.edu/MS-Fit.html). Amino acid side chains are referred to as $R_1$, $R_2$, and so on, in the formulae.

5. One can also examine the spectrum for ion pairs with a mass difference of 28, which could represent *a* and *b* series ions or internal fragments (which can also exit as a pair of ions separated by 28 [*see* **Fig. 4**]). There are two sets of ions that fit this criteria: 117.2–145.8, and 177.3–205.2. Given our interpretation of the sequence from the *y* series ions, these ion pairs correspond to the internal fragment GS and GS-28, and $a_2$ and $b_2$, respectively. The $b_3$ ion is also observed at

292.4. The mass difference between the molecular ion and the $b_3$ ion corresponds to 156.1 + 18, which matches Arg + water. We could also have used dipeptide tables (such as those listed at the WWW site of Burlingame's group at http://rafael.ucsf.edu/MS-Fit.html), which in this case reveal that GS is the only pair with a mass close to that observed, whereas the 205 could be from CT, M(Ox)G, or the correct pair, FG. Therefore, we have been able to assign ions consistent with the putative sequence FGSR using the strategy of observing the immonium ion masses followed by looking for mass differences that correspond to amino acid residues (and pairs of ions separated by either a neutral loss of ammonia [17 u] or water [18 u] or an *a-b* ion series pair [28 u]), in this case, starting with a putative $y_1$ ion.

6. The methyl esterification experiment shown in **Fig. 3B** provides additional confirmation of the sequence interpreted above. The mass of the parent ion only increased from 466.5 to 480.9 corresponding to one methyl group, which would be expected to be added to the C-terminal carboxyl residue. Therefore, all of the *y* series ions would be expected to shift by 14 u, and no other ions should shift compared to unmodified spectrum in **Fig. 3A**.
7. As expected, all of the putative *y*-series fragment-ions have increased in mass by 14 u (boxed in **Fig. 3B**), confirming their identity. None of the other fragment-ions have shifted. When methyl esterification is possible, even if acidic residues are present in the peptide, and therefore increase the parent-ion mass by multiples of 14 u, careful examination of the spectrum can often allow almost complete interpretation of spectrum. An example of when this becomes difficult is when there is one acidic residue and it is at the N-terminus. Then all fragment-ions (except internal fragment ions) are shifted by 14 u, and the parent-ion mass is 28 u higher than the unmodified ion. The immonium ion masses of Glu and Asp are also increased by 14 u following methyl esterification.

### 3.6. Fragment Ion Searches

Search programs, in addition to the peptide-mass search programs (*see* **Subheading 3.4.**), which use either partially or uninterpreted fragment-ion spectra to search protein or translated nucleotide sequence databases, are publicly available on the internet (WWW). The following is a list of servers currently available together with their affiliations and URL addresses. All supply full instructions on-line concerning their use.

1. MOWSE search program from SEQNET, Daresbury: http://www.dl.ac.uk/SEQNET/mowse.html.
2. MS-Tag from the University of California, San Francisco: http://rafael.ucsf.edu/MS-Fit.html.
3. ProFound (and PepFrag) search program from Rockefeller University, New York: http://chait-sgi.rockefeller.edu/cgi-bin/prot-id/1/1.
4. PeptideSearch from EMBL, Heidelberg: http://www.mann.embl-heidelberg.de/Services/PeptideSearch/PeptideSearchIntro.html.

The uninterpreted fragment-ion programs from San Francisco (MS-Tag) and New York (PepFrag) require input of the fragment-ion masses together with the ion series from which they may have been derived (e.g., *a*, *b*, *y*, neutral losses, and so forth). The Daresbury program is not really a fragment-ion search program, but it does allow information to be added to individual peptides in a peptide-mass search, such as how many acidic residues are present (from a methyl esterification experiment) or what amino acid residues are present (which can be derived from immonium ion mass information). The peptide-sequence tag program, which is part of PeptideSearch from Heidelberg, requires at least partial interpretations of the spectrum and assignment of the ions as being either *b* or *y* series. This can sometimes be difficult for PSD-MALDI-MS spectra. However, in the spectrum in **Fig. 3A**, a *y* series tag of (262.0)G(319.7) from a parent of 466.5 could be entered. It should be noted that one would normally use data from a peptide of at least eight to nine residues for database searches. A peptide that is too short will not be as useful in searches of large databases, since too many proteins will have the same (or similar) sequence. With the Heidelberg program, the search can be conducted using both ion series independently, and without constraints on potential modifications on either side of the assigned tag residue(s) (for a complete description, *see* Chapter 55). The program has detailed on-line instructions.

When a result is obtained from any of these search programs, one should attempt to assign all of the ions in the spectrum to the matched sequence. This will allow evaluation of the match and determination of whether any fragment ions are present that are not normally included in the expected ion lists generated by the search software. Regarding the peptide-mass search programs, confidence in the search results can also be gained from evaluation of the scores associated with the top-ranked matches (for those programs that have scores); e.g., if the second-ranked score is considerably less than the top-ranked score, this may be indicative of a good match. However, each program also describes how to evaluate the output. The greatest confidence (and highest score) is achieved when there are a large number of fragment ions observed that match expected ion series.

Of course, if other peptides have been observed from the same protein, even if fragment-ion spectra have not been able to be obtained from them, they can still be used to evaluate the result of the search, e.g., by determining if these other peptides could be derived from the matched protein. One should always attempt to obtain fragment-ion spectra from as many peptides in the mixture as possible to increase one's confidence in the match, and to rule out any possibility of there being more than one protein in a particular band.

## 4. Notes

1. Although we use a column with C8 packing, columns packed with media of differing selectivity may be more appropriate for other applications.

2. When staining the gel, **do not** use a container that has been previously used for immunoblotting protocols, e.g., for blocking with milk, and so on, since even in rinsed containers milk proteins can be adsorbed by either a blot or gel.
3. Trim the protein band carefully so that the gel piece contains only protein.
4. A control gel/membrane piece should always be included in any analysis to allow autodigestion products from the enzyme and any gel/membrane-derived ionizing species to be accounted for.
5. This step is crucial. Complete dryness must be achieved for adequate protein digestion, particularly at low pmol levels.
6. Do not add extra enzyme, since this could lead to increased autolysis and potentially spurious cleavages.
7. Add warm tap water to the sonication bath, and check the temperature prior to sonication.
8. Analysis should be performed as soon as possible, since peptides can be lost owing to adsorption to the microfuge tube, although this seems to be alleviated somewhat with the Immobilon-CD protocol possibly because of the TFA concentration.
9. Just prior to MALDI analysis, a small amount (1–5 µL) of 30% v/v MeCN/ 1% v/v HCOOH can be added to extract any peptides from the walls of the tube.
10. On lower level samples (<2.0 pmol), the majority of recovered peptides can usually (but not always) be found in the digest buffer as opposed to the acidic and organic extracts. Therefore, to increase the possibility of detecting low-level peptides in the respective extracts, these are often pooled and analyzed as one sample on the MALDI-MS. Likewise, it is advantageous to perform the MALDI-MS analysis as soon as possible to avoid again the possibility of peptides adsorbing to the walls of the tubes.
11. When excising the band, try to include only membrane that contains protein by cutting close to the edge of the band.
12. Incubation is conducted at 25°C to avoid the membrane pieces drying out owing to condensation of the buffer solution at the top of the tube. However, this is not a problem if the samples are incubated in a thermostated oven.
13. Separation of the digest buffer is not essential and is not in the original protocol *(4)*.
14. MALDI-mass spectra can be obtained from this sample directly applied to the sample well with matrix, but we have observed stronger signals, presumably owing to partial fractionation of the peptide mixture, if we first use the microcolumn to clean up the sample of salts and contaminants.
15. In addition to equilibrating the column, we suggest that a solution enzymatic digest of a known protein be used to "condition" the column prior to use. That is, load ~5 pmol of an enzymatic digest onto to the microcolumn as described in **Subheading 3.2., steps 7–13**, step-elute the peptides in 30, 70, and 90% v/v MeCN/1% v/v HCOOH, and analyze by MALDI-MS. This will allow the user to become familiar with the use of the microcolumn and potentially block any nonspecific peptide binding sites in the microcolumn. We use the equivalent of a load of 5 pmol, since that is the amount we found to be retained by the C8 microcolumn *(7)*. Therefore, this method is recommended for sample loads of 5 pmol or less.

16. The volume of the wash is sample-dependent. For very dirty/salty samples, wash with an additional 20–30 μL of 1% v/v HCOOH prior to elution, or even use 5% v/v MeCN/1% v/v HCOOH.
17. The step elutions can take place at whatever MeCN concentration you desire. For this example, we use 30, 70, and 90% v/v MeCN/1% v/v HCOOH and a step volume of 3 μL.
18. In most cases, the void/wash sample will give no MALDI data owing to high-salt and contaminant concentration. If peptides are observed, this may be an indication of an overloaded microcolumn, a very old microcolumn, or peptides that do not bind to the column type chosen for the analysis. Also, if additional sensitivity is desired, the 3-μL fractions can be successfully reduced in volume with a gentle $N_2$ stream down to 1.0–0.5 μL.
19. Plasticizers and other contaminants can leech from the tubes over time. Be sure to run matrix only on the target well at various times to determine the mass of any contaminants. In addition, always centrifuge the matrix if there is any particulate matter (i.e., undissolved or precipitated matrix). Otherwise, the intact matrix crystals can act as crystallization seeds, causing inhomogeneous crystal formation *(24)*.
20. Even some small proteins will not ionize with 4HCCA, but will with sinapinic acid, e.g., the phosphoprotein β-casein (~24 kDa).
21. With this approach, it may be necessary to ablate several layers of the calibrant to yield signal from both calibrant and sample.
22. One can also redissolve the crystals in a small amount of additional matrix, or solvent only, if the signal has not improved.
23. The artifactual modifications characterized to date include *(1)*: cysteine-acrylamide (+71 u), oxidized acrylamide (+86 u), β-mercaptoethanol (+76 u); N-terminus acrylamide (+71 u); methionine oxidation (to sulfoxide) (+16 u).
24. It may be possible to rationalize the unassignable masses by taking into account common posttranslational modifications. However, these should always be considered tentative unless confirmatory experimental evidence is obtained.
25. Some confidence in this theory can be gained by determining whether the observed masses can be derived without assuming cleavage specificity of the enzyme (i.e., determining whether a set of contiguous residues sum to the masses observed).
26. Depending on how many masses were obtained, the masses corresponding to the matched protein can be removed and the database searched with this remaining subset of masses. This may even result in identification of the extra protein.
27. This will not occur if the database to be searched is restricted to the species of interest, but can be of assistance if the protein has not been sequenced in your species of interest. This is particularly true when working in genetically poorly characterized species.
28. This is the worst possible outcome, but one that can be interpreted (sometimes) from the difference in the scores between the first- and second-ranked candidates (i.e., if there is little difference in all of the scores, this may be a false positive).
29. Although the ionization by MALDI is said to be relatively soft, it was observed that the intact molecular ions formed undergo significant metastable fragmentation

referred to as postsource decay (PSD) *(25,26)*. This term refers to the fact that the fragmentation is thought to result from multiple early collisions between the analyte (sample) and matrix ions during plume expansion and ion acceleration (i.e., after the source), as well as from collision events in the field-free drift region of the time-of-flight analyzer. Because the metastable fragments, both neutral and charged, have the same velocity as their parent ions, they all reach the linear detector at approximately the same time. The metastable fragments are observed by decelerating ions of discrete energies as a function of their *m/z* ratios with an ion mirror, and then accelerating them back through the field-free flight tube to a second detector. Fragment-ions have a lower kinetic energy than, but although the same velocity as their unfragmented parent ion owing to their smaller mass. These ions are resolved by lowering the potential of the ion mirror (reflector) while maintaining a constant accelerating potential. With a dual-stage reflector, this operation of decreasing the voltage (or scanning of the reflector) is performed between 7 and 14 times to generate a series of spectra that can be concatenated with appropriate software to generate a full-fragment ion spectrum. With the curved-field reflector design of Cornish and Cotter *(27)*, there is no need to step down the voltage, since all of the fragment ions are focused at once, making the process simpler and more rapid. Both types of MALDI-MS instruments have the ability to observe fragment-ion spectra from specific ions in a mixture by only allowing ions of a particular flight time into the field-free drift tube. The resolution of this gating procedure is only about ±2.5% of the parent-ion mass.

30. The predominant ion series observed by PSD-MALDI-MS, after the nomenclature of Biemann *(24)* and Roepstorff and Fohlman *(25)*, are: N-terminal-derived fragments (unimolecular cleavage with charge retained on the N-terminus): *a* and *b*; C-terminal-derived fragments (unimolecular cleavage with charge retained on the C-terminus): *y*; internal (acyl) fragments (two peptide bond cleavages) of the *a* and *b* type: referred to by the sequence of the component residues (with a –28 suffix for the *a* series type); neutral losses of ammonia (17 u, particularly strong if Arg is in the fragment of any series, and may be even more intense than the intact fragment ion) or water (18 u) from any of the previously listed ions depending on the residues contained in the sequence (particularly if Ser or Thr are in the fragment): designated as the ion series with the suffix –17 or –18; and immonium ions: designated as their single-letter abbreviation (*see* **Fig. 4** and **Table 1**). If Pro is present in the peptide, this often results in a strong internal ions series starting at the Pro and extending C-terminal from this site. For example, for a peptide of sequence ILPEFTEAR, a series of PE, PEF, PEFT, and so forth, and PE-28, PEF-28, PEFT-28, and so forth, may be observed. Other internal fragment ions are not unidirectional, but tend to cluster around basic residues present in the peptide. Whether the *a* and *b*, or *y* series ions will predominate the spectrum will depend on which terminus has the strongest basic charge, e.g., if Arg is at the C-terminus, the *y* series ions will probably predominate the spectrum. The strongest immonium ion we have observed is generated by His at 110. Even in its methylated form, as in actin *(28)*, it still yields a strong signal, in this case at 124.

31. More recently Pappin has updated the procedure, and a combination of both procedures that we have tried is presented here *(4)*.
32. Pappin *(4)* recommends using 100-µL glass-tapered vials that have been rinsed briefly in 6 *M* HCl, rinsed thoroughly with $dH_2O$, and dried at 110°C before storing in the presence of desiccant. These vials can be crimp-capped.
33. To ensure no water comes in contact with the solutions, a dry glass syringe stored with desiccant can be used. Any residual water will result in a strong reaction with the thionyl chloride, so it is recommended that all vials and measuring implements be absolutely dry.
34. Although we have generally used only 3× the volume of the original sample, Pappin *(4)* recommends using 10–15 µL of the thionyl chloride solution.

## Acknowledgments

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